PA 168012

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

November 03, 1999

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: ***60/103,153***
FILING DATE: ***October 05, 1998***

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

W. Montgomery

W. MONTGOMERY
Certifying Officer

Please type a plus sign (+) inside this box → [redacted]

Docket Nu[redacted] 1038-856 MIS:ja

PROVISIONAL APPLICATION FOR PATENT COVER SHEET (Large Entity)

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

10/05/98 JC641 U.S. PTO 60/103153 10/05/98

INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
[redacted]	PARRINGTON	45 Martin Street, Bradford, Ontario, Canada, L3Z 1Z4
[redacted] E.	CATERINI	91 Chatfield Drive, Ajax, Ontario, Canada, L1P 2J4

☐ *Additional inventors are being named on page 2 attached hereto*

TITLE OF THE INVENTION (280 characters max)

QUANTITATION OF RNA

CORRESPONDENCE ADDRESS

Direct all correspondence to

☐ Customer Number [ ] → *Place Customer Number Bar Code Label here*

OR

☒ Firm or individual Name	SIM & McBURNEY				
Address	6th Floor				
Address	330 University Avenue				
City	Toronto,	State	Ontario	ZIP	M5G 1R7
Country	Canada	Telephone	(416) 595-1155	Fax	(416) 595-1163

ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification — *Number of Pages* 6

☐ Drawing(s) — *Number of Sheets* [ ] ☐ Other (specify) [ ]

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

☒ A check or money order is enclosed to cover the filing fees — FILING FEE AMOUNT: $150.00

☐ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: [ ]

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are [ ]

Respectfully submitted,

SIGNATURE [signature] DATE October 5, 1998

TYPED or PRINTED NAME Michael I. Stewart REGISTRATION NO. *(if appropriate)* 24,973

TELEPHONE (416) 595-1155

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, DC 20231

P19LARGE/REV04

TITLE OF THE INVENTION

QUANTITATION OF RNA

FIELD OF INVENTION

The present invention relates to the quantitation of RNA present in tissue, thereby permitting analysis of rare transcript expression in cells.

BACKGROUND OF THE INVENTION

It is known in the art how to amplify DNA by polymerase chain reaction (PCR). It has also been shown that mRNA can be transcribed into DNA templates and then amplified by PCR in a method known as RT-PCR. The limitation of existing RT-PCR methods is that some very rare transcripts (mRNAs) are unable to be amplified in the RT and subsequent PCR portion of the existing methods. It would be a great benefit in the analysis of subtle changes in the expression levels of certain genes, for example, cytokines genes, to be able to not only to detect but quantify the levels of these transcripts often alter treatment of the host.

Previously RT-PCR was performed in two distinct steps. This involved the reverse transcriptase step, transcribing mRNA into DNA mediated by the enzyme reverse transcriptase. This enzyme is heat labile so the temperature at which cDNA synthesis was done had to be within a limited temperature range. The next step involved destroying the activity of the RT by heat-inactivation and then adding the DNA polymerase (PCR step) to initiate the amplification phase. By using a recombinant Thermus thermus (rTth) enzyme that possesses both RT activity and DNA polymerase activity,

in the presence of manganese one can reduce and simplify the method to a one-enzyme procedure. Also, using a heat-stable enzyme such as, rTth, one can increase the annealing temperature during the RT stage to increase the specificity of the priming, ensuring only the target RNA sequence is transcribed into DNA. This then greatly increases the ability to amplify the target DNA in the next step, the PCR reaction.

Methods of isolating mRNA are known, for example Graham et al., (J. Immunol., Vol. 151, No. 4, pp 2032-2040) The yields from these methods tend to be very low. When the desired target is a very rare message, these types of isolation procedures do not yield enough total RNA to include the very rare messages. Therefore, it is desirable to improve the yield of the total mRNA from various tissue samples so the rare messages are included. Also if the yields of mRNA from certain tissues, for example, lungs, are increased, this reduces the need to pool several samples from different animals, thereby reducing the biological diversity. This allows the researcher to define what is happening in a specific animal after treatment.

To investigate the expression levels of rare messages, it is important to extract from the tissue of interest enough intact total message to enable amplification and quantitation of these rare messages.

SUMMARY OF THE INVENTION

The present invention relates to the determination of RNA production in cells. By modifying existing procedures, the invention permits an accurate quantitation of small quantities of RNA, representative of rare transcripts in cells, in particular the quantitation of cytokine RNA in mouse lung and spleen tissue.

RNA isolation is effected using TRIzol Reagent (GIBCO/BRL) a mono-phasic solution of phenol and guanidine isothiocyanate. During sample homogenization or lysis, TRIzol Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an organic phase, an interphase layer containing DNA and protein contaminants, and an aqueous phase, where the RNA is exclusively located.

After separation of the aqueous phase, the RNA may be separated therefrom by precipitation through mixing with isopropyl alcohol or other suitable alcohol and centrifugation. The supernatant is removed and the RNA pellet is washed once with ethanol followed by air drying the pellet.

In accordance with the present invention, this conventional RNA isolation procedure is improved by washing the homogenization probe between samples in the following manner. The probe first is washed with sterile water and ethanol and then twice with sterile water using wash tubes. The probe is wiped off with a sterile wipe both after the initial and final sterile water wash before proceeding with the homogenizing of the first tissue samples. This washing operation removes residual ethanol and prevents sample carryover.

Reverse transcriptase PCR amplification of the RNA is next carried out via a single enzyme reaction using rTh DNA polymerase and appropriate probes. The use of such recombinant enzyme permits higher temperatures for RT incubation, which leads to more specific binding and thus an accurate amplification.

The gene segments used as probes depend on the RNA under investigation. For example, for cytokines, the applicants have identified the following gene segments:

Murine 1L-4: na 249 to 363 (SEQ ID Nos. 1, 2)
Murine 1L-5: na 336 to 402 (SEQ ID Nos. 3, 4)
Murine 1L-10: na 401 to 495 (SEQ ID Nos. 5, 6).
Murine IFN-γ: na 404 to 507 (SEQ ID Nos. 7, 8)

Determination of the quantity of RNA in the specific tissue sample is possible using any convenient tag, such as the PE ABI 7700 fluorescence detection system and Taqman chemistry. The use of a fluorescence detection system permits quantitation of RNA in tissue down to a level of 100 molecules.

The procedure of the present invention enables an improved yield and purity of RNA to be obtained in comparison to published data, such as in the Graham et al reference referred to above. Rather than tissue from two mice being necessary to obtain sufficient RNA for amplification, individual mice can be analyzed and over 200 assays are possible for each RNA sample.

Whole tissue is analyzed as opposed to RNA extracted from cells, as described in Anduse et al. above.

SEQUENCES

Mouse IL-4 sequence (sense strand (SEQ ID No: 1) with anti-sense (SEQ ID No: 2) below):

CGTCCTCACA GCAACGAAGA ACACCACAGA GAGTGAGCTC GTCTGTAGGGC TCCAAGGTGC
50

TTCGCATATT TTATTTAAAA CATGGGAAAA CTCCATGCTT GAAGAAGAAC TCT - SEQ ID No. 1
113

GCAGGAGTGT CGTTGCTTCT TGTGGTGTCT CTCACTCGAG CAGACATCCC GAAGGTTCCA
60

CGAAGCGTAT AAAATAAATT TTGTACCCTT TTGAGGTACG AACTTCTTCT TGAGA - SEQ ID NO. 2
115

Mouse IL-5 sequence (sense (SEQ ID No: 3) with anti-sense (SEQ ID No: 4) below):

ACCGCCAAAA AGAGAAGTGT GGCGAGGAGA GACGGAGGAC GAGGCAGTTC CTGGATTACC
60

TGCAAGA - SEQ ID No: 3
67

TGGCGGTTTT TCTCTTCACA CCGCTCCTCT CTGCCTCCTG CTCCGTCAAG GACCTAATGG
60

ACGTTCT - SEQ ID No: 4
67

Mouse IL-10 sequence (sense strand (SEQ ID No: 5) with anti-sense (SEQ ID No: 6) below).

TGAATTCCCT GGGTGAGAAG CTGAAGACCC TCAGGATGCG GCTGAGGCGC TGTCATCGAT
60

TTCTCCCCTG TGAAAATAAG AGCAAGGCAG TGGAG - SEQ ID No. : 5
95

ACTTAAGGGA CCCACTCTTC GACTTCTGGG AGTCCTACGC CGACTCCGCG ACAGTAGCTA
60

AAGAGGGGAC ACTTTTATTC TCGTTCCGTC ACCTC - SEQ ID No. : 6
95

Mouse interferon-gamma mRNA sequence (sense strand (SEQ ID No: 7) and anti-sense (SEQ ID No: 8) below):

CATTCATGAG TATTGCCAAG TTTGAGGTCA ACAACCCACA GGTCCAGCGC CAAGCATTCA 60

ATGAGCTCAT CCGAGTGGTC CACCAGCTGT TGCCGGAATC CAGC - SEQ ID NO. : 7 104

GTAAGTACTC ATTAACGGTT CAAACTCCAG TTGTTGGGTG TCCAGGTCGC GGTTCGTAAG 60

TTACTCGAGT AGGCTCACCA GGTGGTCGAC AACGGCCTTA GGTCG - SEQ ID No. : 8 105

SUMMARY OF THE DISCLOSURE

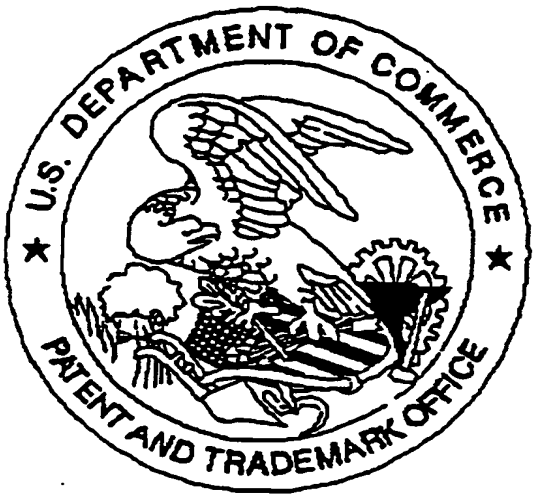
In summary of this disclosure, the present invention provides an accurate manner of determining the quantity of RNA in cells, to permit analysis of rare transcripts, such as cytokines, based on a modified RNA isolation procedure, RT-PCR in a single enzyme reaction and fluorescence detection. Modifications are possible within the scope of this invention.

PRINT OF DRAWINGS
AS ORIGINALLY FILED

PRINT OF DRAWINGS
AS ORIGINALLY FILED

United States Patent & Trademark Office

Office of Initial Patent Examination -- Scanning Division



SCANNING INSTRUCTIONS

Application deficiencies were found during scanning:

☐ Page(s)_____ of Declaration _____ were not present for scanning.
(Document title)

☐ Page(s)_____ of _____ were not present for scanning.
(Document title)

☐ Scanned copy is best available.